Uncoupling of endothelial nitric oxide synthase in perivascular adipose tissue of diet-induced obese mice

Ning Xia, Sven Horke, Alice Habermeier, Ellen I. Closs, Gisela Reifenberg, Adrian Gericke, Yuliya Mikhed, Thomas Münzel, Andreas Daiber, Ulrich Förstermann, Huige Li

Materials and Methods

Animals

C57BL/6J mice are a well-established mouse model of diet-induced obesity. Male C57BL/6J mice were put on high-fat diet (ssniff® EF D12492 II, 60% energy from fat; Soest, Germany) for 20 weeks starting at the age of 8 weeks. Control animals were fed normal control diet (NCD; ssniff, 11% energy from fat). At the age of 28 weeks, the animals were sacrificed and analyses performed. The animal experiment was approved by the responsible regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz; 23 177-07/G 1-021) and was conducted in accordance with the German animal protection law and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Assessment of vascular function

Thoracic aortas were isolated and dissected into rings of 2-3 mm in length, with perivascular fat and connective tissues either removed or left intact. Isometric tension was recorded using a Wire Myograph system (Danish Myo Technology, Aarhus, Denmark). The rings were equilibrated for 60 minutes and contracted two times with 120 mM KCl. For assessment of vascular function, the rings were pre-contracted with norepinephrine to reach the submaximal tension (80% of that obtained with 120 mM KCl), before vasodilation was induced by acetylcholine (ACh).

Immunohistochemistry studies

Thoracic aorta samples (with PVAT) were fixed in buffered 4% formaldehyde and then embedded in paraffin. Four-micron-thick cross sections of the aorta were stained with a rabbit polyclonal antibody against eNOS (NB300-500, Novus Biologicals; Cambridge, UK) using the VECTASTAIN ABC Kit (Vector Laboratories; Burlingame, CA). Briefly, the primary eNOS antibody was biotinylated and incubated with the
tissue specimens, followed by incubation with streptavidin-peroxidase. The reaction was visualized with 3,3′-diaminobenzidine.

**Direct assessment of NO release**

NO in PVAT was detected with 4,5-diaminofluorescein diacetate (DAF-2 DA), a cell-permeable derivative of the fluorescent NO probe DAF-2 that is hydrolyzed to DAF-2 by intracellular esterases. DAF 2-DA can be used in fluorescence microscopy to measure real-time changes in NO levels.

For a better comparison, NCD and HFD cryostat sections were mounted pairwise back-to-back on the same slide. This eliminates potential artefacts otherwise caused by non-uniform staining of the sections. After an incubation with or without the NOS inhibitor N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME, 500 \(\mu\)M), the slides were loaded with DAF-2 DA (20 \(\mu\)M) in the absence or presence of acetylcholine (100 nM). Then, fluorescence imaging was immediately performed by real-time time-lapse imaging in a heated (37°C) incubator chamber with a Zeiss 710 confocal laser scanning microscope (Zeiss, Germany). DAF-2 DA was excited at 488 nm and fluorescence emitted between 495 nm and 550 nm was collected.

**Measurement of ROS production**

PVAT-derived ROS production was measured with two independent methods: L-012 chemiluminescence and electron paramagnetic resonance (EPR). PVAT samples from thoracic aorta were pre-incubated in the presence or absence of 500 \(\mu\)M L-NAME for 30 minutes. Then, 100 \(\mu\)M of the luminol derivate 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazino-1,4-(2H,3H)dione sodium salt (L-012) was added, and chemiluminescence measurement was performed after a dark adaptation of 30 min. For detection of superoxide with EPR, the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethyl-pyrrolidine hydrochloride (CPH, 1 mM) was used. Paired measurements were performed with two PVAT samples from the same aorta segment, one in the absence and one in the presence of PEG-SOD (100 U/ml; Sigma-Aldrich). The SOD-inhibitable portion of EPR signal was considered as superoxide production.

**Gene expression analyses**

RNA was isolated using peqGOLD TriFast™ (PEQLAB) and cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real time RT-PCR (qPCR) reactions were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich) and 20 ng cDNA. Relative mRNA levels of target genes were quantified using comparative threshold \(C_T\) normalized to housekeeping gene TATA-binding protein (TBP).

mRNA expression in control animals with NCD were set 100%. The qPCR primer sequences were as follows:
eNOS_forward: CCT TCC GCT ACC AGC CAG A, eNOS_reverse: CAG AGA TCT CTA CTG CAT TGG CTA; GCH1_forward: TGG TGA TTG TGA AGG ACA TAG ATA TG, GCH1_reverse: GCC AAT ATG GAC CCT TCC TAC A; DHFR_forward: AAT CCT AGC GTG AAG GCT GGT A, DHFR_reverse: GGC GAC GAT GCA GTT CAA T; arginase 1_forward: GGA ACC CAG AGA GAG CA T GA, arginase 1_reverse: TTT TTC CAG CAG ACC AGC TT; arginase 2_forward: ACC AGG AAC TGG CTG AAG TG, arginase 2_reverse: TGA GCA TCA ACC CAG ATG AC; TBP_forward: CTT CGT GCA AGA AAT GCT GAA T, TBP_reverse: CAG TTG TCC GTG GCT CTC TTA TT.

Western blot analyses

Western blot analyses were performed with total protein samples (30 µg each) from aorta or PVAT. The following primary antibodies were used: rabbit monoclonal antibody against β-tubulin I (catalog number T7816, Sigma-Aldrich; 1:200,000), mouse monoclonal antibody against eNOS (catalog number 610297, BD Transduction Laboratories; 1:2000), mouse monoclonal antibody against GCH1 (catalog number H00002643-M01, Abnova; 1:500), mouse monoclonal antibody against DHFR (catalog number 610697, BD Transduction Laboratories; 1:500), rabbit monoclonal antibody against arginase I (catalog number ab124917, Abcam; 1:2000), rabbit polyclonal antibody against arginase II (catalog number ab154422, Abcam; 1:1000). Western blot was carried out as previously described 2, 3. Protein samples were separated on a Bis-Tris gel and transferred to a nitrocellulose membrane. Blots were blocked in 5% milk powder in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.1% Tween 20) for one hour at room temperature. The primary antibodies were diluted in the same solution used for blocking at 4°C overnight. Blots were then washed in TBST and incubated with a horseradish peroxidase-conjugated secondary antibody diluted in 5% milk in TBST for one hour. After washing in TBST and then in TBS, the immunocomplexes were visualized using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) according to the manufacturer’s instructions. Densitometric analysis of scanned blots was performed using the Quantity One software (Bio-Rad).

Measurement of biopterins and L-arginine

Tetrahydrobiopterin (BH₄), dihydrobiopterin (BH₂) 3, 10 and L-arginine 11 were measured by using high-performance liquid chromatography (HPLC)-based methods, as previously described.

For BH₄ measurement, aorta and PVAT samples were homogenized in ice-cold lysis buffer (0.1 mM Tris-HCl, pH 7.8, containing 5 mM ethylenediamine tetraacetic acid, 0.3 mM KCl, 5 mM 1,4-dithioerythritol, 0.5 mM Pefabloc, and 0.01% saponin) with 25 nM neopterin as internal standard. Samples were oxidized under either acidic conditions (with 0.2 M HCl containing 50 mM I₂) or alkaline conditions (with 0.2 M NaOH containing 50 mM I₂). The biopterin content was assessed by HPLC with fluorescence detection (350-nm
excitation, 450-nm emission). BH₄ concentration was calculated as femtomol per microgram of protein by subtracting the biopterin peak resulting from alkaline oxidation (accounting for BH₂) from the biopterin peak resulting from acidic oxidation (accounting for both BH₂ and BH₄).³¹⁰

For L-arginine measurement, samples were lysed in 400 µl ice-cold ethanol 70% for 4 min at 50 Hz with a QIAGEN TissueLyser. Two nmol N⁶-monomethyl-L-arginine (L-NMMA) were added as internal standard, then supplemented with 0.9 ml PBS pH 7.4 and applied to an Oasis MCX ion exchange column (Waters, Eschborn, Germany). The column was washed with 1 ml each, 0.1 N HCl and methanol, and subsequently cationic amino acids (CAA) were eluted with 1 ml methanol:water:25% NH₃ (5:4:1), vacuum dried and resuspended in 0.2 ml sodium borate buffer (0.5 M, pH9.6). For precolumn derivatization, 50 µl sample was supplemented with 12 µl OPA reagent (100 mg o-phthaldialdehyde), 9 ml methanol, 1 ml borate buffer (0.5 M), 100 µl 2-mercaptoethanol, and 18 µl acidic acid (1 M). Amino acid derivatives (10 µl) were separated on a Nova-Pak column (C18, 4 µm 3.9 x 300 mm, Waters, Eschborn, Germany) using a gradient of 50 mM sodium acetate (pH 6.8 supplemented with 0.044 % TEA) and acetonitril. The flow rate was 0.8 ml/min. Fluorescence (excitation wavelength, 330 nm; emission wavelength, 450 nm) was monitored with a RF-20 A fluorometer (Shimadzu) and quantified using the analysis program McDAcq (Bischoff). Recovery of CAA was about 88-92 %.

Statistics

Results are expressed as mean ± SEM (standard error of the mean). Student’s t test was used for comparison of HFD group with NCD group. Two-way ANOVA was used to compare the curves. P values < 0.05 were considered significantly different. For statistical analysis GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used.

References


